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14. ABSTRACT

Tumor phenotype is influenced by multiple factors, including genetic and epigenetic alterations, tumor stroma, and systemic environment. Because of this complexity, it has been difficult to investigate the influence of normal cell phenotype on the behavior of its tumorigenic derivatives. In our previously published work we have shown that 10 BPLER cells were capable of forming tumors in mice and these BPLER tumors were metastatic to the lungs. In contrast, 100,000 HMLER cells derived from the same individuals were required to form tumors in mice and these tumors were non metastatic. The results of our studies during the first year of the grant confirm that genetic differences between BPLER and HMLER cells can not account for the phenotypic differences between these tumor types. The initial round of epigenetic studies with chromatin modifying drugs as well as Chip-on-chip analysis of H3K79me2 indicates that there is a significant difference between BPLER and HMLER cells derived from the intrinsic differences between their normal sell-of-origins.

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Introduction:

Tumor phenotype is influenced by multiple factors, including genetic and epigenetic alterations, tumor stroma, and systemic environment. Because of this complexity, it has been difficult to investigate the influence of normal cell phenotype on the behavior of its tumorigenic derivatives. We developed a cell culture method that allows direct comparison of two genetically matched tumors derived from two different breast epithelial cell types. This revealed that tumor cell phenotype, including metastatic tendency and gene expression profile, can be strongly influenced by their respective normal cell-of-origin in this experimental model. This observation raises the question of whether some of the clinical differences observed among subtypes of human breast cancers can be traced to their respective normal in vivo cells-of-origin. This is a new concept, and the question can not be easily answered with existing technology. Thus, using new methods we have developed, we are mapping the cell-type-specific pre-existing epigenetic pattern of normal breast epithelial sub-populations in our experimental model in order to develop tools that can be applied to human tumor samples [1].

Body:

During the first year of the grant period we have completed a number of the experiments that were proposed under task 1. In our previously published work we have shown that 10 BPLER cells were capable of forming tumors in mice and these BPLER tumors were metastatic to the lungs. In contrast, 100,000 HMLER cells derived from the same individuals were required to form tumors in mice and these tumors were non metastatic [1]. The primary goal of these experiments was to determine whether this difference in tumorigenicity between the two cell lines is due to genetic differences. If this were the case one might expect that BPLER cells could be genetically more unstable and accumulate more genetic alterations. Alternatively, the added aggressiveness of BPLER cells could have been due to gain of a particular oncogene or

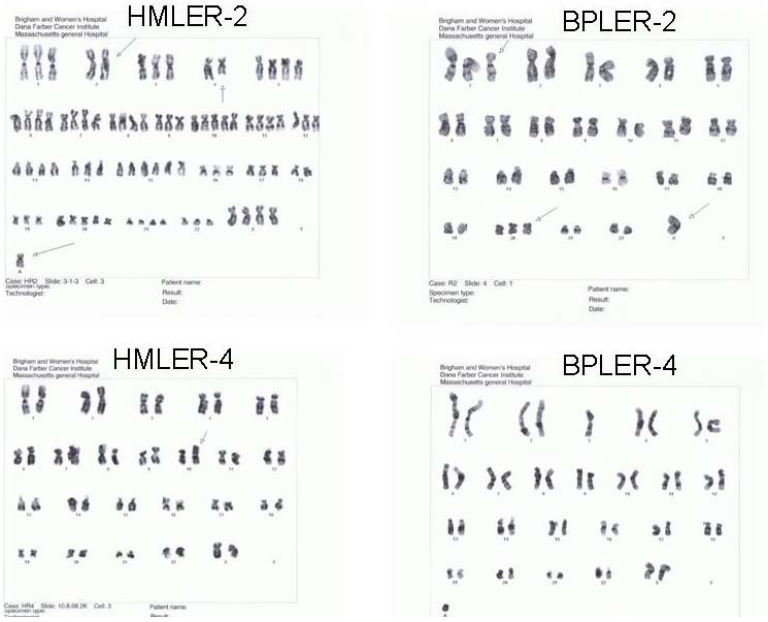
loss of a particular tumor suppressor. In this such an oncogene is case one might expect that all three BPLER lines would share a common amplification or deletion in the region of the genome that correspond to such a gene. The experiments that address these questions were originally proposed in task 1, and the preliminary results of these experiments are described below.

Statement of work:

Task 1a) *Examine the genetic alterations in BPEC, HMEC populations, their transformed derivatives, and FACS isolated CD44 (+) and (-) subpopulations with GTC banding, CGH and SNP arrays (months 1-3).*

1) Karyotype Analysis: We have completed the karyotype analysis of nine cell lines corresponding to two different normal breast cell types isolated from three different individuals (BPE 2, HME2, BPE3, HME3, BPE4, HME4) and their tumorigenic derivatives (BPLER2, HMLER2, BPLER3, HMLER3, BPLER4 and HMLER4) (Figure1). We did not find any karyotype changes (deletions, amplifications and translocations) that occurred in BPLER cells but not in HMLER cells.

Figure 1 Karyotype analysis of two different normal breast cell subtypes a (*BPE* and *HME*) and their transformed derivatives (*BPLER* and *HMLER*)



The results in Figure 1 indicate that the difference in tumorigenicity between the two breast cell types – BPLER and HMLER- is unlikely to be due to a difference in their tendency to accumulate genetic alterations, because the over all frequency of abnormal karyotypes were similar between the cells. Furthermore, there was a great heterogeneity of the karyotypes within in each tumor cell line among different individual cells (Table 1); for example examination of four different **HMLER-4** karyotypes showed four different karyotypes [(**1**) 45,X,i(10)(q10),del(18)(p10), (**2**) 88<4n>,XXXXX,-7,-9,-11,-12,-15,-18,-19,-22,+6mar, (**3**) 46,XX,i(10)(q10) and (**4**) 45,XX,add(5)(p?),-6] and examination of seven different **BPLER-4** karyotypes showed no repeating consistent changes either [(**1**) 43,XX,del(10)(p?),-11,-17,-19, (**2**)46,XX,-3,+mar, (**3**)47,XX,-5,-8,i(10)(q10),+3mar, (**4**)49,XX,-1,+5,+7,+9,+13, (**5**)87<4n>,del(1)(p?),-2,-4,+8,-10, der(10;13)(q10;q10),-13,-16,-19,+20,-22,+2mar, (**6**)46,XX,del(1)(q10),i(8)(q10),add(14)(p?), add(22)(q?), (**7**)97<4n>,XXXXX,+1,+3,-5,+7,-8,i(8)(q10),-9,-10,+12,-13,+19,+20,+21,+22]. **This result indicates that the difference in tumorigenicity is not due to selection of rare mutant subclones during the transformation process**.

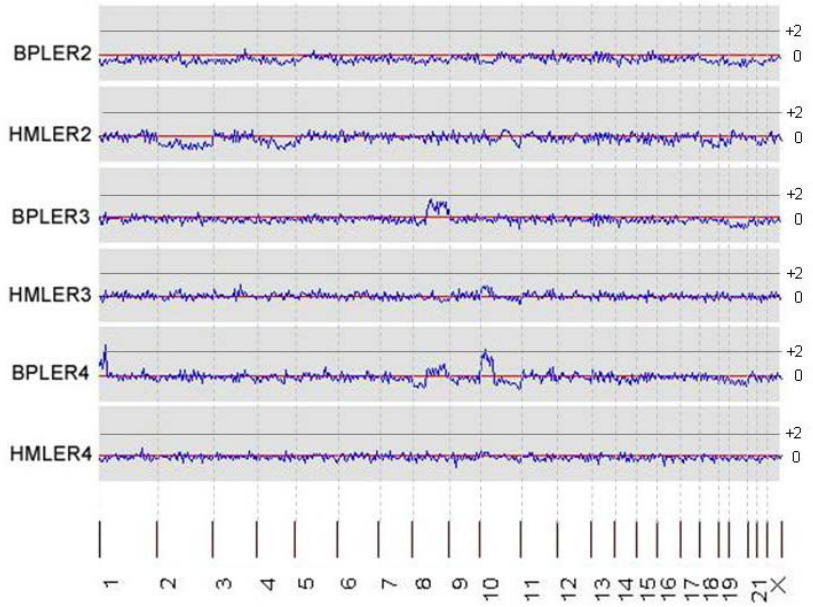
Table1 Karyotype analysis of two different normal breast cell subtypes a (*BPE* and *HME*) and their transformed derivatives (*BPLER* and *HMLER*)

Cell Line	Karyotype
HME2	46,XX *(6 cells)* 45,XX, der(10;15)(q10;q10)
HME4	46,XX,i(10)(q10) *(4 cells)* 47,XX,+3
BPE2	46,XX,-5,-10,-18,del(10)(p?)add(14)(p?),+3mar 92<4n>, XXXX, -1,-3,+4,-8,-9,-12,+13,-14,-15,+16,-18,+20,-21,+22,+6mar 46,XX, -5,-10,-18, +3mar 46,XX, -4, +3, -8, -10, -13, +14, +20, +2mar
BPE4	46,XX,add(1)(p?),-21,+mar 48,XX,+9,add(1)(p?) Partial—46,XX,add(1)(p?),i(10)(q10),+6mar 185<4n>,XXXX,-10,-12,+20,i(10)(q10),+mar 47,XX,-1,-5,+7, del(5)(p?),i(10)(q10), del(11)(q?), add(13)(q?),+2mar 93<4n>,XXXX,+3,-5,-6,+7,-8,+13,-14,-15,+17,+20,+21,+22
HMLER2	83<4n>,XXXX,-2,del(4)(q?),-7,-10,-15,-16,-18,-19,-22,+mar 87<4n>,XXXX,-2,-4,-5,-6,-7,-8,-9,-13,-17,-18,+19,+20,+21,-22,+4mar 88<4n>,XXXX,-2,+3,-4,-6,-8,-9,-11,-16,-18,-19,+21,+7mar 83<4n>,XXXX,-2,-4,-6,-10,-14,add(16)(p?),-18,-19,-22,+2mar 82<4n>,XXXX,-1,-2,-3,-4,del(4)(p?),-9,+10,-12,-14,+15,-16,-17,-18,-19,+20,-22,+mar 77<4n>,XXXXX,+1,-2,-3,-4,-5,-6,-8,-10,-11,-13,-15,-16,-17,-18,-19,-20,-21,+7mar
HMLER4	45,X,i(10)(q10),del(18)(p10) 88<4n>,XXXXX,-7,-9,-11,-12,-15,-18,-19,-22,+6mar 46,XX,i(10)(q10) 45,XX,add(5)(p?),-6
BPLER2	46,XX,del(8)(p?),-14,-18,-21,+3mar 89<4n>,XXXX,-1,-6,+7,-9,del(10)(p?),-13,-15,+20,-21,+mar 47,XX,+20 47,X,+1,del(1)(p?),+20
BPLER4	43,XX,del(10)(p?),-11,-17,-19 46,XX,-3,+mar 47,XX,-5,-8,i(10)(q10),+3mar 49,XX,-1,+5,+7,+9,+13, 87<4n>,del(1)(p?),-2,-4,+8,-10, der(10;13)(q10;q10),-13,-16,-19,+20,-22,+2mar 46,XX,del(1)(q10),i(8)(q10),add(14)(p?),add(22)(q?) 97<4n>,XXXXX,+1,+3,-5,+7,-8,i(8)(q10),-9,-10,+12,-13,+19,+20,+21,+22

2) SNP Array Analysis: We have also analyzed the genomic DNA of the above cells with a SNP array. While the karyotype analysis is able to reveal numeric alterations in chromosome number and large scale deletion, amplification and translocations, it is not possible to detect small changes in the genomic DNA with this method. Thus, SNP arrays which have a much higher resolution were used to exclude the possibility that the difference in the phenotype of BPLER vs. HMLER tumors is due to small genetic alterations. We extracted genomic DNA from HME, BPE, HMLER and BPLER cells and hybridized to SNP arrays. In Figure 2 we show that the vast majority of the chromosomes in the tumorigenic cells do not contain deletions compared to their normal cell-of-origin. And the occasional gains such as in chr. 8 in BPLER-3 cells and chr. 10 in BPLER-4 cells are less than 2-fold in magnitude and are only present in one of three BPLER lines. These data corroborate the karyotype results and once again indicate that the difference in the phenotype of BPLER vs. HMLER tumors is unlikely to be due to genetic differences.

Conclusion: The total number of genetic alterations were similar between BPLER and HMLER cells, and no recurring amplification, deletion, or translocation is detected that is uniformly present in all three independently derived BPLER lines but not in their HMLER counterparts (and vise versa). Thus, we did not detect a consistent genetic difference that is a likely candidate which can explain the phenotypic difference between BPLER vs. HMLER cells.

Figure 2. Genomic DNA analysis of BPLER and HMLER cells normalized pair wise to their respective cell-of-origin cell BPE or HME hybridized to SNP arrays

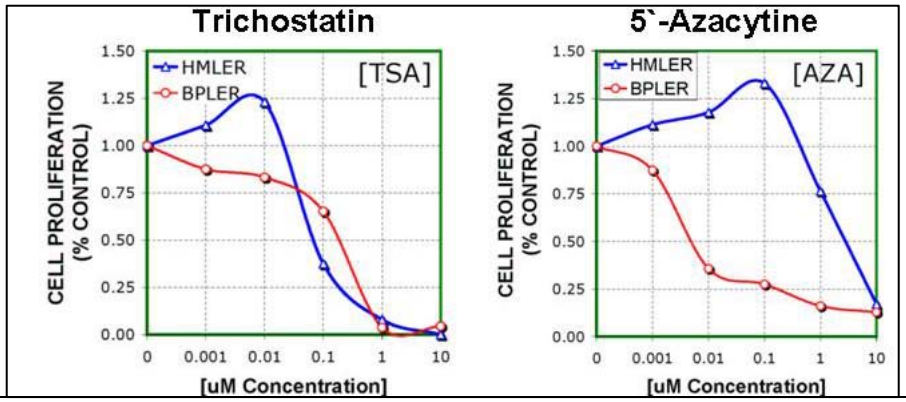


Task 1b, e) *Compare sensitivity of normal (BPEC vs. HMEC) and tumorigenic (BPLER vs. HMLER) cells to histone deacetylase (HDAC) inhibitors and DNA methylation inhibitors (months 1-6).*

In preliminary experiments we have found that there is a nearly three orders-of-magnitude difference in the sensitivity of BPLER vs. HMLER cells to growth inhibitory effects of DNA methylation inhibitor 5`-Azacytidine (Fig. 3) and also a significant difference in their response to low levels of HDAC inhibitor TSA (Fig. 3). Interestingly, the BPLER cell cultures, with a high frequency of tumor-initiating cells, were much more sensitive to this treatment than HMLER cells and conventional tumor cell lines; suggesting that this type of treatment may be effective against tumor stem cells. Furthermore, this difference in sensitivity between the BPLER and HMLER tumor cells appear to be inherited from their normal cell-of-origin as expected; BPEs were also much more sensitive than HMEs to 5-Aza and TSA. Lastly, the difference in sensitivity was maintained even when both tumor cells types were grown in the same medium, thus it was a difference in the cells and not just medium conditions.

Conclusion: These results suggest that there is a significant epigenetic difference between BPLER and HMLER cells both at the level of DNA methylation and at the level of histone acetylation.

Figure 3. Inhibition os cell proliferation with HDAC inhibitor trichostatin-A (TSA) and DNE methyl transferase inhibitor 5-Azacytidine (AZA)



Task 1 c-d) Compare the global histone modification patterns and DNA methylation pattern differences between normal (BPEC vs. HMEC) and tumorigenic (BPLER vs. HMLER) cells with ChIP-on-Chip assays (months 1-12).Compare the global histone modification patterns and DNA methylation pattern differences between FACS-isolated CD44-high tumorigenic and CD44-low non-tumorigenic BPLER tumor initiating cells with ChIP-on-Chip assays (months 12-24).

1) Identifying Genes Differentially Expressed Between BPLER and HMLER Cells at the Transcriptional Level

We carried out ChIP-on-Chip assays as described [2], with genomic DNA prepared from BPE2, BPLER2, HME2 and HMLER2 cells. In these experiments fragments of genomic DNA that are bound to Histone H3K79me2 is captured *in vivo* by formaldehyde crosslinking, followed by cell lysis, DNA fragmentation and immunoaffinity precipitation of the DNA fragments that are bound to a specific protein using an antibody that specifically recognizes H3K79me2. The precipitated DNA was then purified, PCR amplified and sequenced for identification of the genomic region that is bound to a particular transcription factor. In order to examine whole-genome view of protein-DNA interactions chromatin immunoprecipitation (ChIP) is combined with DNA microarray analysis, known as ChIP-on-Chip.

2) Processing and analysis of the ChIP-chip data:

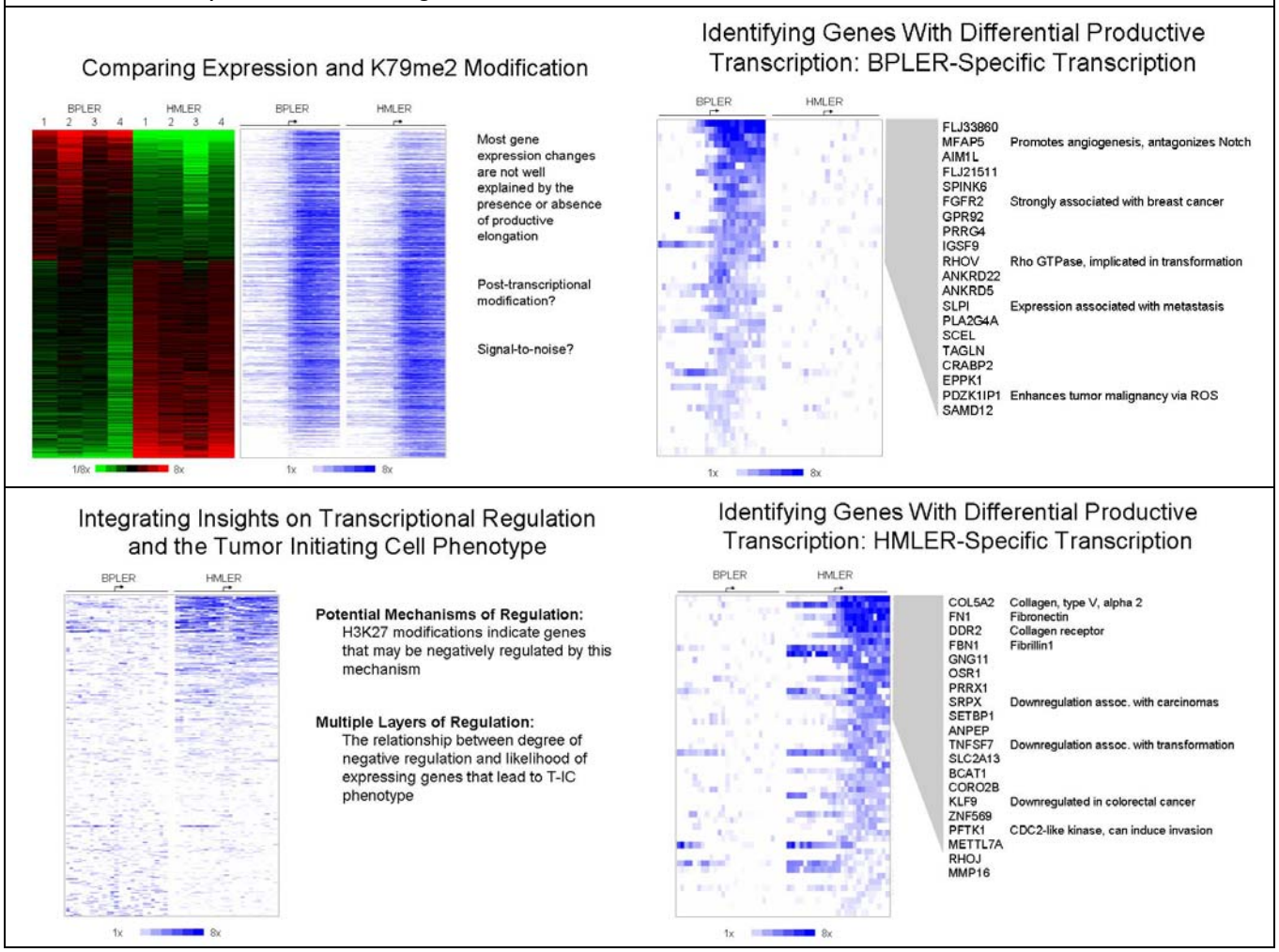
a) We selected probesets from expression data with a 2x or greater change in expression between BPLER and HMLER cells using criteria from Ince et al. 2007 (Supp. Table 4) [1]**,** which yielded this gives 4726 probesets, which are limited to those that map to EntrezGene ID's and have corresponding ChIP-chip data.

b) Of these 4726 probesets, 4205 can be mapped uniquely to an EntrezGene ID (3109 genes).
Of these 4205 probesets, 4024 were represented on our promoter arrays for ChIP-chip.
Of these 4024 probesets, 3096 correspond to a transcription start site where we have sufficient ChIP-chip data (post-processing for cluster analysis). These probesets map to 2277 genes.
To prepare the ChIP data for cluster analysis, we carried out a binning process. For each transcription start site, we set up a series of bins, usually between 250 and 350 bp in size, that extend both upstream and downstream of the start site. Probes on the array are then assigned to one or more bins based on their distance from the start site. This allows us to smooth out the distribution of the data to make it possible to cluster and visualize.

An example of this analysis is included in Figure 4. In brief, H3K79me2 modification correlates with a transcriptionally active gene. Our results show that only a subset of the genes that differentially expressed at the transcript level are differentially modified at the H3K79me2 between BPLER and HMLER. This result suggests to us that the difference in the mRNA levels of many genes between BPLER and HMLER is not due to chromatin modification and active transcription. Thus, filtering the gene expression data with the Chip-on-chip analysis allowed us to identify the most relevant transcriptional differences between HMLER and BPLER cells (Figure 4). An ontological examination of the top 10 % of the BPLER specific transcripts revealed that they are involved in 1-Developmental process, 2-Anatomical structure development, 3-Multicellular organismal development, 4-Intercellular junction system development, 5-Apical junction development, 6-Organ development, 7-Apicolateral plasma membrane, 8-Ectoderm development, 9-Tissue development, 10-Anatomical structure morphogenesis, and 11-Plasma membrane. In contrast, the top 10% of HMLER transcripts are all involved in proteinaceous extracellular matrix interestingly.

Conclusion: These results illustrate the proof of principle for our proposal. We have demonstrated that when ChIP-on-chip technology is used in combination with gene expression profiles it can identify the subset of genes that are transcriptionally regulated. There are more than 4,000 transcripts that are differentially expressed between BPLER and HMLER cells. By examining the H3K79me2 profile of the same genes we determined that approximately 10% these transcripts are regulated at the transcriptional level. Hence, with this type of analysis the number of candidate genes we have to work with is narrowed down by 10-fold, which will potentially allow us to verify their expression with RT-qPCR and develop functional assays to test selected candidate genes for their role in explaining the cell-of-origin difference between BPLER and HMLER cells.

Figure 4. Chip-on-chip analysis of BPLER and HMLER cells normalized pair wise to their respective cell-of-origin cell BPE or HME.



Key Research Accomplishments:

We have successful completed most of the experiments that had been proposed in task 1 including:

1) Karyotype analyses
2) SNP array analyses
3) Chip-on-chip optimization and analyses

Reportable Outcomes:

We're preparing RO1 applications based on work supported by this award; and plan to submit these applications during the 2009/2010 grant cycle.

Conclusion:

The results of our studies during the first year of the grant confirm that genetic differences between BPLER and HMLER cells can not account for the phenotypic differences between these tumor types. The initial round of epigenetic studies with chromatin modifying drugs as well as Chip-on-chip analysis of H3K79me2 indicates that there is a significant difference between BPLER and HMLER cells derived from the intrinsic differences between their normal cell-of-origins.

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1) Ince TA, Richardson.A., Bell, GW, Saitoh M, Iglehart JD, Weinberg RA, *Transformation of distinct human breast epithelial cell types leads to different tumor phenotypes.* Cancer Cell, 2007 **12**(2): p160-70.
2) Lee, T.I., S.E. Johnstone, and R.A. Young, *Chromatin immunoprecipitation and microarray-based analysis of protein location.* Nat Protoc, 2006. **1**(2): p. 729-48.